2019

Survey of Biosynthetic Gene Clusters from Sequenced Myxobacteria and Analysis of Potential Metabolic Diversity

Katherine C. Gregory
University of Mississippi

SURVEY OF BIOSYNTHETIC GENE CLUSTERS FROM SEQUENCED MYXOBACTERIA AND ANALYSIS OF POTENTIAL METABOLIC DIVERSITY

by
Katherine C. Gregory

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2019

Approved by

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Advisor: Dr. D. Cole Stevens

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Reader: Dr. Dale George Nagle

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Reader: Dr. Ziaeddin Shariat-Madar

ABSTRACT

The importance of natural product discovery to society is undeniable as natural products have seemingly infinite applications, particularly in regard to pharmacological use as antibacterial, antifungal, antiparasitic, anticancer and immunosuppressive agents. The search for new therapeutics and other new chemical commodities thus grows simultaneously with antibiotic resistance and commercial pressure on pharmaceutical research. However, the increasingly common phenomenon of natural product rediscovery continues to inhibit advancement in this field (1). We contend that the likelihood of rediscovery can be predicted by taxonomic distance between the bacteria in question and previously studied bacteria. That is, our data supports a correlation between chemical diversity and taxonomic distance, and we offer the hopeful perspective that less commonly explored genera have outstanding potential for unique natural product discovery. Specifically, we have examined the order *Myxococcales*, a reputable source of secondary metabolites. Our findings have been facilitated by the mining of bacterial genomes for biosynthetic gene clusters (BGCs), a methodology that has become a critical component to natural product discovery as it takes advantage of the increasing number of sequenced bacterial genomes available through public databases. By using the BiG-SCAPE-CORASON platform to generate sequence similarity networks that contain 994 BGCs identified by antiSMASH from 36 sequenced myxobacteria, we have observed that every predicted BGC was specific to one of three current suborders of myxobacteria. The analysis of BGCs detected within four additional, draft genomes supports the observation that myxobacterial biosynthetic diversity correlates with taxonomic distance and suggests the likelihood of rediscovery when targeting previously investigated genera. Further, 822

BGCs with no notable homology to characterized clusters within the MIBiG database are presented. This survey portrays the biosynthetic diversity of these BGCs and exemplifies the potential for natural product discovery from myxobacteria. Herein we report the likelihood for rediscovery of known metabolites from bacteria belonging to previously explored genera. The results depict significant biosynthetic potential of bacteria associated with overlooked taxa within the *Myxococcales* and possibly other natural product-associated bacteria orders.

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LIST OF ABBREVIATIONS

antiSMASH	antibiotics & Secondary Metabolite Analysis Shell
BGC	biosynthetic gene cluster
BiG-SCAPE	Biosynthetic Gene Similarity Clustering and Prospecting Engine
CORASON	CORe Analysis of Syntenic Orthologues to prioritize Natural products biosynthetic gene clusters
GCF	gene cluster family
MIBiG	Minimum Information about a Biosynthetic Gene cluster
NRPS	nonribosomal peptide synthetases
RiPPs	ribosomally synthesized and post-translationally modified peptides
t1PKS	type I or modular polyketide synthases

I. INTRODUCTION

Microbial secondary metabolites

Microbial natural products are chemical substances produced by bacteria. The natural products that are the focus of this study are known as secondary metabolites, because they are produced as a result of secondary metabolism and are not necessary for the survival of the producing organism but are known to have other biologically advantageous functions for the organism in its natural environment. In contrast, primary metabolites, such as amino acids, nucleotides, lipids and carbohydrates, are requisite for the growth of bacteria.

Secondary metabolites, biosynthesized from structurally simple compounds like amino acids or glucose, elicit a great amount of scientific attention because of their complexity and immense chemical diversity (2). Many secondary metabolites have wide ranges of molecular weights, and many exhibit great stereo-complexity, often having a high number of stereogenic carbon centers (3). In other words, there is a significant number of derivatives that can potentially be generated from these natural products.

Microbial secondary metabolites amount to around 50,000 known compounds with the two of the more noteworthy contributors being streptomycetes and myxobacteria (4, 5). Actinomycetes, the family under which streptomycetes falls, is generally noted to be the frontrunner for microbial natural product producers, but myxobacteria have started to receive more recognition for being competitive contributors of secondary metabolites (6).

Myxobacteria

Bacterivorous myxobacteria, predatory microorganisms ubiquitous to aquatic and soil sediments, employ a variety of social and predation strategies, such as the formation of fruiting bodies and spores, cooperative swarming, and collective predation on other bacteria as well as fungi (7). These relatively sophisticated biological activities not only make them effective micropredators but also prolific providers of diverse secondary metabolites. In fact, over 100 bioactive secondary metabolites have been discovered from many of the taxa within the entire order *Myxococcales,* which comprises three described suborders, 10 families, 29 genera, and 58 species as of June 2018 (8, 9). Dawid reported that between 50 and 100% of myxobacteria produce bioactive compounds (10). This coupled with the fact that the genome of myxobacteria is around double the size of *Escherichia coli* and around the same as that of the genus *Streptomyces,* the potential for drug discovery through the exploration of myxobacteria is seemingly quite promising (1).

Traditional methods of natural product discovery

The methodologies and strategies for natural product discovery have evolved greatly over the last century. The first natural product brought to the market was penicillin during WWII, but the real search for natural products began with and resulted from the Waksman group at the Rutgers lab in the 1940s as they screened actinomycetes from soil samples and subsequently discovered actinomycin, streptothricin, and, most significantly, streptomycin (3). Pharmaceutical companies began funding research whose focus was screening environmental samples of actinomycetes and fungi and testing isolated products against test organisms. After acquiring samples containing microorganisms then completing the process of detection, fermentation and isolation of secondary metabolites,

researchers performed phenotypic screening to test the harnessed products for bioactive capabilities (3).

Several advancements were made starting in the 1970s and continued into 1990s that made this field of research much more efficient, like combinatorial biosynthesis and certain target-based approaches (3). Yet, despite such developments and the subjects of studies being microorganisms that are generally known to produce bioactive secondary metabolites, the results from the aforementioned method of bioactivity-based screening have been discouraging in recent years, which is primarily attributed to the tendency of this type of approach to rediscover known natural products (11). In fact, rediscovery of known compounds has been proclaimed "the bane of natural products discovery" (3). Researchers were motivated to explore other means by which to find novel secondary metabolites, leading to the popularity of modern genomics-based approaches.

Genomics in natural product discovery

Genome-sequencing technology has become widely accessible and inexpensive in recent years, leading to a steady increase in the number of bacterial genomes and biosynthetic gene clusters (BGCs) publicly available through online databases. Medema and coworkers define a BGC as "a physically clustered group of two or more genes in a particular genome that together encode a biosynthetic pathway for the production of a specialized metabolite (including its chemical variants)" (12). It has become apparent through microbial genomics that many microorganisms possess cryptic BGCs, gene clusters that encode proteins that control the synthesis of certain secondary metabolites but that are not active under laboratory growth conditions (13). By scanning DNA sequences, the structures of new secondary metabolites can be predicted, which is

especially useful in regard to cryptic BGCs that will not produce these compounds without special activation (3).

Three powerful and complimentary computational tools for BGC identification and analysis are antiSMASH (antibiotics & Secondary Metabolite Analysis Shell), BiG-SCAPE (Biosynthetic Gene Similarity Clustering and Prospecting Engine), and CORASON (CORe Analysis of Syntenic Orthologues to prioritize Natural products biosynthetic gene clusters) (14, 15). The antiSMASH software tool identifies and predicts BGCs, which are then used by BiG-SCAPE to determine gene cluster families (GCFs) and generate a sequence similarity network based on these families with the help of the MIBiG (Minimum Information about a Biosynthetic Gene cluster) repository that contains a large collection of identified BGCs (14, 15). Sequence similarity networks are used to depict the relationships among protein sequences, clustering sequences with a certain level of homology that can be preemptively specified if desired. Following BiG-SCAPE analysis, CORASON can be used to perform a phylogenetic analysis that shows the evolutionary relationships of BGCs within each GCF (15). The workflow for how these software tools all work together is depicted in Fig. 1.

A genomics-driven analysis of myxobacteria

Here we present a survey of all myxobacterial natural product BGCs that are included in the antiSMASH database and provide documentation of all BGCs with and without characterization and assigned metabolites, illustrating the capacity for discovery from sequenced myxobacteria. A homology network that contains 994 BGCs from 36 sequenced myxobacteria was constructed with the use of the BiG-SCAPE/CORASON platform (15). Edges between BiG-SCAPE's rendered GCFs indicate shared domain

types, sequence similarity, and similarity of domain pair-types amongst input BGCs (15). A comparative analysis against the MIBiG repository (v1.4) demonstrates the great amount of BGCs that have not yet been characterized (12).

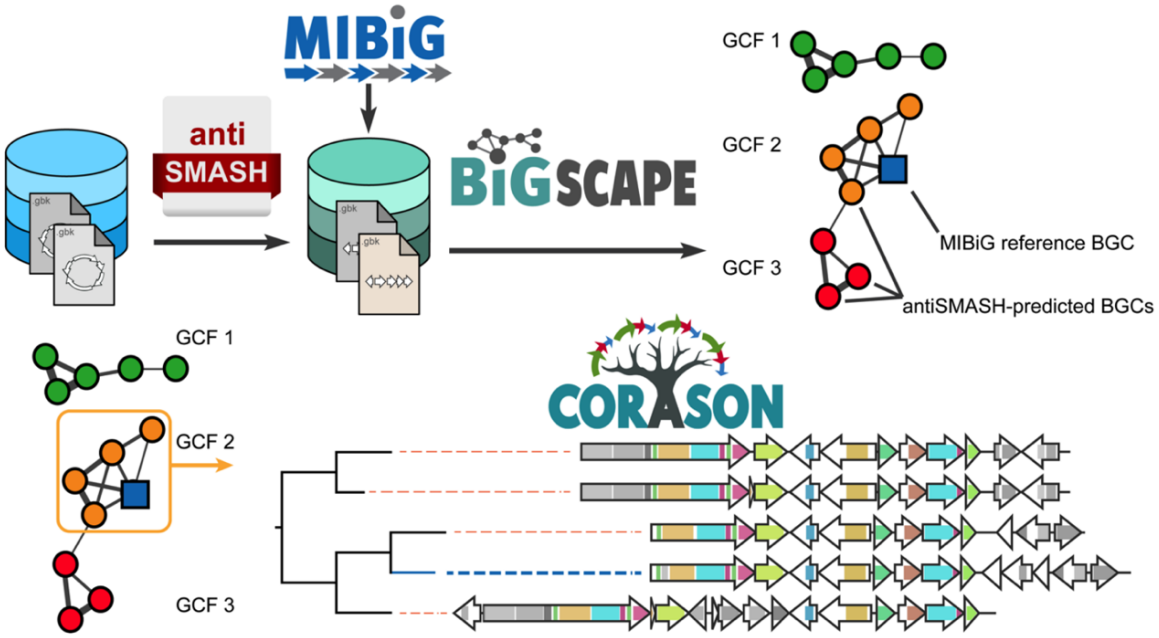

Fig 1. The BiG-SCAPE/CORASON workflow (15).

II. RESULTS AND DISCUSSION

BiG-SCAPE analysis of BGCs from sequenced myxobacteria

Using BiG-SCAPE, we constructed a sequence similarity network that shows homology across 735 GCFs, containing 994 total BGCs as unique nodes from 36 myxobacteria and includes 1,035 edges (Fig. 2). A total of 613 singletons without significant homology using a similarity cutoff of 0.30 were also included in the network to appropriately depict all myxobacterial BGCs within the antiSMASH database (14, 16). Predicted BGC classes included 64 type I or modular polyketide synthases (t1PKS), 57 PKS categorized by antiSMASH as "PKSother" that includes all non-modular categories of PKSs, 125 nonribosomal peptide synthetases (NRPS), 166 hybrid PKS-NRPS, 245 ribosomally synthesized and post-translationally modified peptides (RiPPs), 149 terpenoid clusters, three saccharide clusters, and 185 clusters not belonging to any of the aforementioned classes that antiSMASH categorizes as "Others" clusters (14, 17). While hybrid PKS-NRPS pathways that include both PKS and NRPS domains are organized into a specific separate grouping, all other hybrid pathways that include more than one BGC are categorized in the Others class (14, 15). The Others-associated BGCs included clusters with 133 predicted products as well as 52 hybrid BGCs (Fig. 3). This breadth of biosynthetic diversity from just 36 myxobacteria includes 23 out of 44 BGC-types currently designated by antiSMASH (14, 17).

Discovered metabolites from myxobacteria and associated BGCs

Of the 994 BGCs analyzed, 149 possess sequence similarities greater than 75% with annotated BGCs in the MIBiG repository (v 1.4) (12). As these BGCs produce characterized metabolites or potentially analogues thereof (Fig. 4A), a total of 85% of the

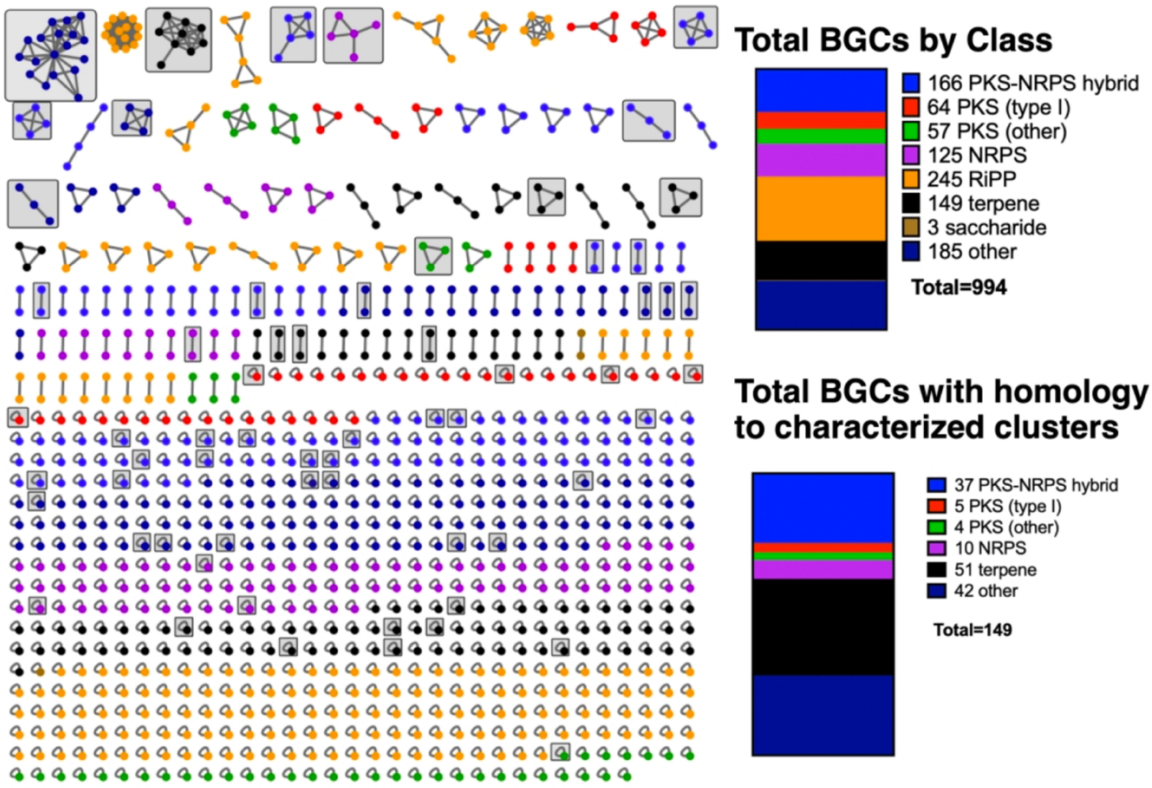

Fig. 2 Sequence similarity network of 994 myxobacterial BGCs deposited in the antiSMASH database generated by BiG-SCAPE and rendered with Cytoscape. All GCFs that include at least one BGC with sequence similarity greater than 75% to a characterized cluster deposited in the MIBiG repository are boxed in grey (excluding 23 geosmin BGCs). Totals for BGC class diversity and GCFs (including geosmin BGCs) with homology to MIBiG clusters and color reference provided (right).

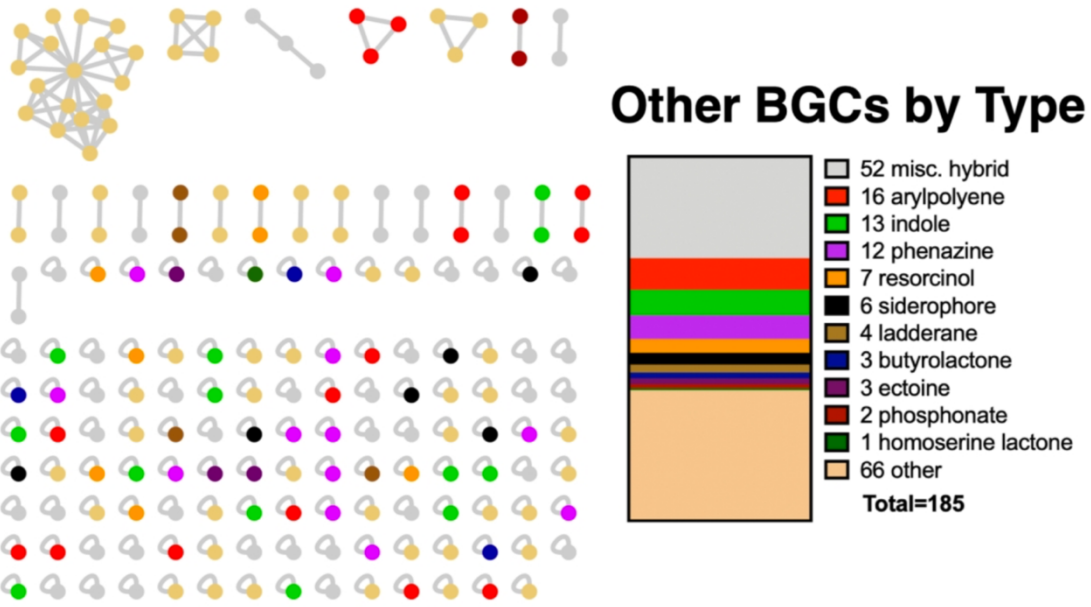

Fig. 3 Sequence similarity network of myxobacterial BGCs classified as Others in the antiSMASH database with predicted product type and totals (right).

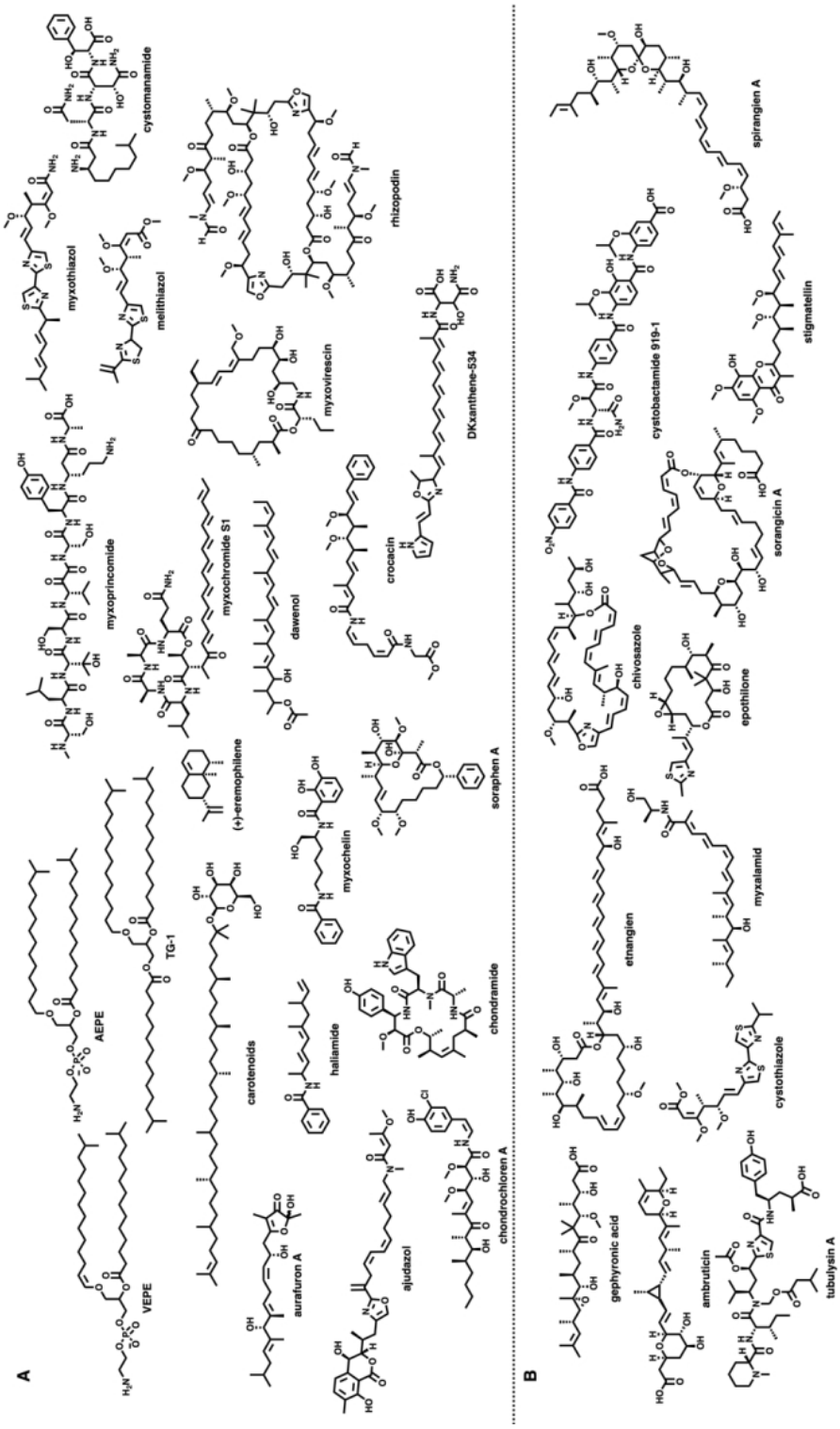

Fig. 4 Secondary metabolites associated with BGCs determined to possess significant sequence similarities to characterized clusters in MIBiG (A) and metabolites associated with known BGCs from myxobacteria with lower sequence similarities to BGCs included in the dataset with (B).

BGCs within the network produce yet to be discovered metabolites (18-42). Considering the range in quality across the 36 total genomes and draft genomes incorporated in the antiSMASH database, we also considered additional BGCs with similarity scores lower than 75% that had similarities with MIBiG clusters reported from myxobacteria identified by antiSMASH. This analysis provided an additional 23 BGCs that might produce metabolites with overlapping chemical diversities to the products delineated within the MIBiG repository (Fig. 4B) (43-57). Including this inference, 83% of the BGCs within the network lack any association with a reported myxobacterial metabolite. The biosynthetic diversity of these mapped BCGs includes five t1PKS, 10 NRPS, 37 hybrid PKS-NRPS, four PKSother, 28 terpenoid clusters, and 42 Others (Fig. 2). While the vast majority of BGCs were considered singletons or unclustered individual nodes without sequence similarity to other analyzed BGCs, GCFs with more than one member BGC often shared sequence similarities with characterized MIBiG clusters. Interestingly, BGCs with high sequence similarity to specific MIBiG clusters were not always assigned the same cluster class nor included within an individual GCF. For example, nine GCFs that include a single BGC with high homology to the myxochelin BGC were assigned as NRPS, hybrid PKS-NRPS, and Others type clusters (32, 33). Trees generated by CORASON provide the phylogenetic diversity associated with these myxochelin BGCs (Fig. 5) (15). Analysis of these trees indicated that such wholesale affiliation with each of these GCFs led to inclusion of BGCs that were in fact not related to the myxochelin BGC but instead shared proximal similarity to a BGC within the family that also included a neighboring mxyochelin-like BGC (Fig. 5) (32, 33). While this assuredly leads to an

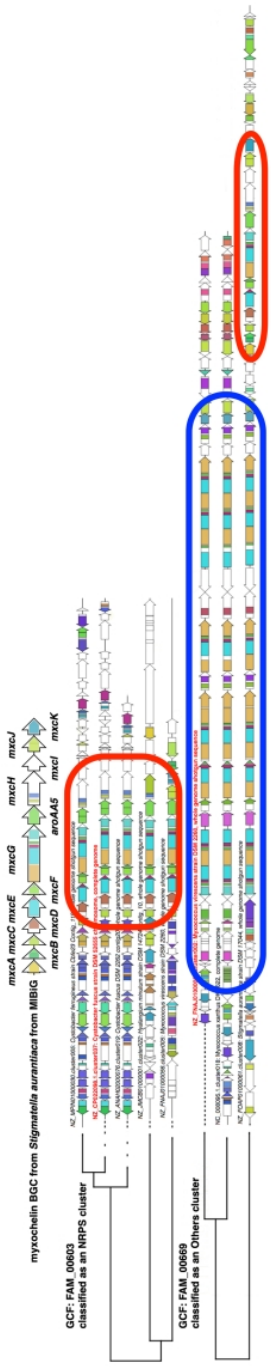

Fig. 5 Phylogenetic tree of BGCs included in NRPS GCF (FAM_00603) and Others GCF (FAM_00669) generated by CORASON with sequence similarities to the myxochelin BGC in MIBiG (BGC0001345) boxed in red and the sequence similarity leading to clustering of FAM_00669 boxed in blue. The BGCs boxed in blue are omitted from our estimate of unexplored biosynthetic space due to sequence similarities between the myxochelin BGC and one BGC within the GCF. This omission typifies our conservative estimate of uncharted myxobacterial BGCs.

underestimation of the unexplored biosynthetic space as presented, this only supports our eventual conclusion that a vast wealth of biosynthetic space from these myxobacteria remain unexplored. Other BGCs observed across multi-member GCFs included: 26 BGCs within 11 GCFs homologous to a carotenoid cluster from *Mxyococcus xanthus*, 24 BGCs and four GCFs associated with the characterized VEPE/AEPE/TG-1 biosynthetic pathway from *M. xanthus* DK1622, and 11 BGCs across five GCFs with significant similarity to the hybrid PKS-NRPS DKxanthene cluster (18, 19, 23, 24, 42). While all of the BGCs included in this charted biosynthetic space might not correlate to the corresponding metabolites associated with each MIBiG cluster, we consider this a rigorous assessment that provides a conservative estimate of outstanding, uncharacterized BGCs and remaining opportunity for natural product discovery.

Biosynthetic space with undiscovered metabolites

Perhaps the most obvious absence in the 172 BGCs associated with characterized BGCs, no RiPP clusters with significant sequence similarity to MIBiG clusters were observed (58-60). However, the recently reported RiPP, crogacin A produced by *Chondromyces crocatus* is the only myxobacterial RiPP discovered to date, and the BGC is currently not deposited in the MIBiG repository (61). Also of note, crogacin A possesses a unique tetracyclic structure not associated with other classes of RiPPs (61). This combined with the 245 uncharacterized BGCs within our network suggests that myxobacteria to be an excellent resource for the discovery of RiPPs with novel chemical scaffolds. While accounting for far fewer BGCs within the network, no sequence similarities were observed for the three saccharide BGCs that include the aminoglycoside and aminocyclitol subtypes (62-64). All other BGCs considered unexplored accounted for the

vast majority of BGCs within each cluster class including: 92% of t1PKS, 98% of PKSother, 92% of NRPS, 81% of terpenoid clusters, 78% of hybrid PKS-NRPS, and 77% of Others. Interestingly, within the BGCs assigned to the Others class three butyrolactone and one homoserine lactone clusters were identified. Specialized metabolites belonging to these types of clusters are quorum-signalling molecules produced by Streptomyces and numerous non-myxobacterial Proteobacteria respectively (65-68). Although putative quorum signal receptors have been observed in myxobacterial genomes, no metabolite associated with either of these quorum signalling systems nor regulatory process attributed to either's presence has been reported from a myxobacteria (69).

IV. EXPERIMENTAL

Dataset

All BGCs associated with the order *Myxococcales*, a total of 994 BGCs from 36 myxobacteria, were obtained from downloaded as .gbk files from the antiSMASH database (https://antismash-db.secondarymetabolites.org) (14).

BIG-SCAPE-CORASON analysis

BiG-SCAPE version 20181005 (available at: https://git.wageningenur.nl/medema-group/BiG-SCAPE) was used locally to analyze the 994 BGCs as individual .gbk files downloaded from the antiSMASH database (1/30/2019) (14, 15). BiG-SCAPE analysis was supplemented with Pfam database version 31 (70). The singleton parameter in BiG-SCAPE was toggled to ensure that BGCs with distances lower than the default cutoff distance of 0.3 were included in the corresponding output data. The MIBiG parameter in BiG-SCAPE was toggled to include the MIBiG repository version 1.4 of annotated BGCs (12). The hybrids-off parameter was toggled to prevent hybrid BGC redundancy. Generated network files separated by BiG-SCAPE class were combined for visualization using Cytoscape version 3.7.1; annotations associated with each BGC were included into Cytoscape networks by importing curated tables generated by BiG-SCAPE (16). All BGCs with sequence similarities to deposited MIBiG clusters greater than 75% were indicated and annotated using Cytoscape (16).

V. CONCLUSIONS

The potential benefits associated with the discovery of novel, biologically active bacterial metabolites are clear considering their possible applications as antimicrobials or anticancer therapeutics. With the insight provided by the excess of available genome data from sequenced microbes and the assistance of newly developed and publicly available software tools, the search for new bacterial natural products can be steered in the right direction toward biosynthetic space that is largely yet to be explored and that belongs to gifted producers of secondary metabolites, namely myxobacteria. The vast discrepancies between the BGCs of myxobacteria with sequence similarity to characterized pathways and BGCs without lend significant credence to the likelihood of continued discovery of novel metabolites from just this subset of 36 myxobacteria and exemplifies the outstanding potential associated with the *Myxococcales* at large.

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